

A New Class of Phytoestrogens: Evaluation of the Estrogenic Activity of Deoxybenzoins

Nikolas Fokialakis,¹ George Lambrinidis,¹
Dimitra J. Mitsiou,² Nektarios Aligiannis,¹
Sofia Mitakou,¹ Alexios-Leandros Skaltsounis,^{1,*}
Harris Pratsinis,⁴ Emmanuel Mikros,¹
and Michael N. Alexis^{2,3,*}

¹Laboratory of Pharmacognosy and
Pharmaceutical Chemistry
Department of Pharmacy
University of Athens Panepistimiopolis Zografou
GR-15771 Athens

²Molecular Endocrinology Programme

³Biomedical Applications Unit
Institute of Biological Research and Biotechnology
National Hellenic Research Foundation
GR-11635 Athens

⁴Laboratory of Cell Proliferation & Ageing
Institute of Biology
NCSR “Demokritos”
GR-15310 Athens

Summary

Although deoxybenzoins are intermediates in the synthesis of isoflavones, their estrogenic activity has not been investigated. Eleven deoxybenzoins were synthesized and their estrogenicity was evaluated. While their affinities for estrogen receptors (ER) ER α and ER β were found grossly comparable to those of daidzein, some exhibited considerable selectivity and transcriptional bias toward ER β , which appeared to allow for enhancement of ER-mediated transcription via deoxybenzoins binding of ER β . Their activity to stimulate the proliferation of ER-positive breast cancer cells and regulate the expression of endogenous and stably transfected reporter genes differed considerably, with some inhibiting cell proliferation while effectively inducing gene expression at the same time. Molecular modeling confirmed that deoxybenzoins fit well in the ligand binding pocket of ER β , albeit with different orientations. Our data support the view that deoxybenzoins constitute a promising new class of ER β -biased phytoestrogens.

Introduction

Estrogens of plant origin (phytoestrogens) are known to affect the physiology of the female reproductive system (uterus, ovaries, breast), the cardiovascular system, the brain, and the skeleton. Consequently, there is growing interest in using phytoestrogens and synthetic derivatives thereof for the prevention of several diseases, including breast and prostate cancer, postmenopausal osteoporosis, and cardiovascular diseases [1, 2]. Phytoestrogens are believed to exert their physiological effects via the estrogen receptor (ER), a ligand-activated

regulator of transcription. Two subtypes of ER have been described, ER α and ER β , and reported to exhibit overlapping but distinct tissue distribution patterns and to differ in their ligand binding ability and transcription activation properties [3, 4]. Some phytoestrogens have been reported to act as ER β -selective modulators of transcriptional activation and/or repression [4, 5]. Both ER subtypes have a modular structure comprising distinct functional domains associated with hormone binding, DNA binding and transcriptional activation [3]. The C-terminal ligand binding domain (LBD) is known to play a crucial role in unraveling the activity of antiestrogens as well as estrogens, as demonstrated by the recently published X-ray structures of the LBD of ER α and ER β with 17 β -estradiol (hereafter referred to as estradiol), raloxifene, 4-hydroxytamoxifene, DES, or genistein [6–8]. In the agonist-bound conformation, an interactive surface is created that is capable of recruiting a variety of coactivators. By contrast, in the antagonist-bound conformation, the LBD is known to recruit corepressors [3, 5, 9].

Deoxybenzoins, intermediates in the synthesis of isoflavones, are found in several plant and marine sources [10, 11]. Plants with known estrogenic activity in their extracts, such as *Glycyrrhiza sp.* [12] *Trifolium subterraneum* [13] and *Ononis spinosa*, are good sources of natural deoxybenzoins. Although the estrogenic activities of *Glycyrrhiza sp.* and *Trifolium sp.* have been attributed mainly to isoflavones [14], their structural similarities with deoxybenzoins led us to study the estrogenic activity of the latter. Here, we report on the synthesis and the estrogenic activity of 11 (among them three new) deoxybenzoins and discuss the structural determinants of their interaction with ER β .

Results and Discussion

Chemical Synthesis

Using a method previously described by Wahala et al. [11], a number of deoxybenzoins were synthesized. Starting from the appropriately substituted phenols and phenylacetic acids, a Friedel-Crafts reaction catalyzed by boron trifluoride takes place (Figure 1A). Among the dihydric phenols, resorcinol and substituted resorcinols react with variously substituted phenylacetic acids, giving excellent yields of deoxybenzoins. In contrast, the trihydric phenol phloroglucinol gave good yield by reacting with p-methoxy-phenylacetic acid but not with p-hydroxy-phenylacetic acid, despite that different conditions were tested. For this reason, compound 4 was obtained by the demethylation of compound 3 using boron tribromide [15] in dichloromethane at -70°C . In the reactions of p-hydroxy-phenylacetic acid with resorcinols, minor byproducts with an ethoxy group in the 4' position were detected. It is known [16] that ethyl fluoride is produced from the complex of boron trifluoride with ethyl ether, which can react with the hydroxyl group of p-hydroxy-phenylacetic acid. In the case of 4-chloro-

*Correspondence: mnalexis@eie.gr (M.N.A.), skaltsounis@pharm.uoa.gr (A.-L.S.)

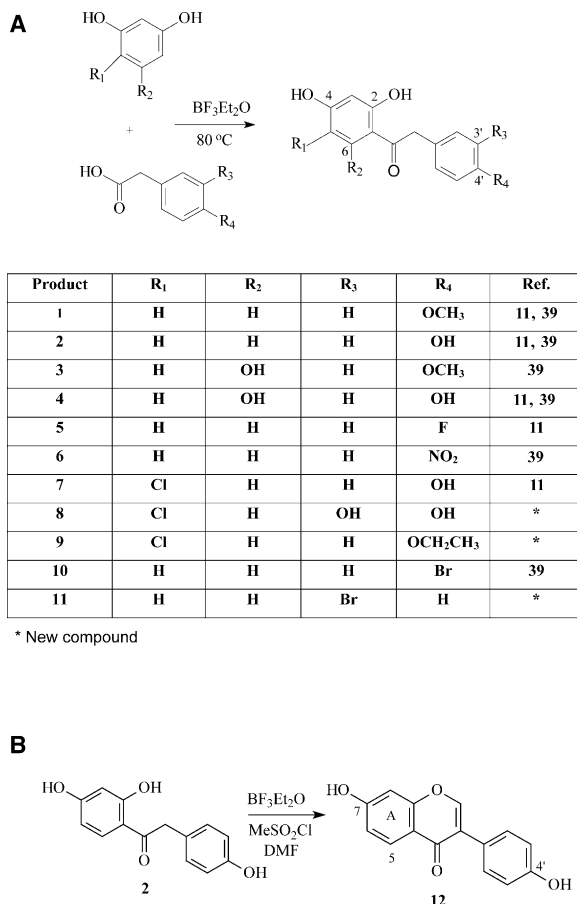


Figure 1. Synthesis of Deoxybenzoins and Daidzein

(A) Synthesis of deoxybenzoins 1–11.

(B) Synthesis of daidzein.

resorcinol, compound **9** was obtained in yield of 12%. Isoflavone daidzein (**12**) was obtained by the cyclization of the corresponding deoxybenzoins (**2**) using boron trifluoride as catalyst (Figure 1B) [11].

Relative Binding Affinities for ER α and ER β

Deoxybenzoins binding of ER α and ER β was studied using fluorescence polarization (FP) to determine the concentration (IC₅₀) required for 50% inhibition of ES2 (fluorescent ER ligand) binding to commercial receptor preparations [17]. The relative binding affinity (RBA) values of deoxybenzoins for ER α (RBA α) thus determined were comparable to that of the isoflavone daidzein (**12**) (Table 1). By contrast, RBA β values varied considerably, with that of **2** in particular being ~ 15 -fold higher than those of daidzein and the natural deoxybenzoins ononetin (**1**). Notably, **2** exhibited by far the highest selectivity for ER β ; **5**, **6**, **10**, and **11** were moderately ER β -selective; **1**, **3**, **4**, **8**, **9**, and daidzein exhibited a moderate bias toward ER α ; and only **7** was unbiased. Inspection of the structures of Figure 1 reveals that ER β selectivity is associated with R₁, R₂, R₃ = H and R₄ = OH, F, or NO₂ (**2**, **5**, **6**), and that it may reflect a better accommodation of Br bulkiness (**10**, **11**).

Deoxybenzoins Activation of Transcription via ER α and/or ER β

To discern how deoxybenzoins affect ER α and ER β transcriptional activity, we transfected HEK-293 human embryonic carcinoma cells with an expression vector for human ER α or ER β as well as a luciferase reporter endowed with a consensus estrogen-responsive element (ERE) and the core thymidine kinase (tk) promoter. Using 0.1–0.4 μ g of ER expression vector and 1 nM estradiol, we observed maximum reporter responses amounting to 16.3 ± 0.5 - and 10.8 ± 0.9 -fold in the presence of ER α and ER β , respectively. However, because ER α exhibited higher constitutive transcriptional activity than ER β , hormonal inductions were 5.3 ± 0.4 - (ER α) and 5.8 ± 0.3 -fold (ER β). By comparison, using **2** at 10 μ M, at which concentration all estrogenic deoxybenzoins exhibit maximum proliferative response (see Figure 3B), inductions were 6.1 ± 0.1 - (ER α) and 9.2 ± 0.2 -fold (ER β) (Figure 2A), indicating a more effective recruitment of coactivators to the tk promoter by deoxybenzoins binding of ER β . Thus, ER β -mediated deoxybenzoins induction of the response is higher than the ER α -mediated one and, in addition, exceeds that of the hormone significantly (ANOVA, $p < 0.01$). The IC₅₀ values for ER α - and ER β -mediated inductions by **2** of a response equal to that of the hormone were 421 and 66 nM, respectively. This 6.4-fold difference in potency exceeds the ER β binding selectivity of **2** by ~ 1.2 -fold, implying again that deoxybenzoins binding may render ER β more capable of recruiting transcriptional coactivators to the tk promoter than ER α . Table 1 (columns 5 and 6) compares reporter inductions at 10 μ M deoxybenzoins to those at 10 μ M daidzein and 1 nM estradiol in the presence of either ER α or ER β . Only **2** exhibited higher reporter responses than estradiol. Interestingly, several deoxybenzoins, whether ER β selective or not, exhibited a bias toward ER β -mediated transcription at 10 μ M, with **1**, **4**, **5**, **7**, **9**, and **11** apparently being the most biased. Table 1 shows further that daidzein binding of ER α and ER β provided for somewhat higher reporter responses than **2**, albeit slightly less biased.

In light of the above, we wondered whether the ER β binding selectivity and transcriptional bias of **2** affects reporter responses in the presence of both ER. We co-transfected HEK-293 cells with ER α (0.1 μ g) and ER β (0.1 or 0.2 μ g) expression vectors, keeping vector amounts well below the limit (0.5 μ g) we have observed squelching effects to come about with these cells, and exposed transfectants to 30 and 100 nM **2**, concentrations that provide for ER β -mediated inductions of 3.0- and 4.5-fold, but ER α -mediated inductions of 1.3- and 2.1-fold, respectively (Figure 2A). We observed that 100 μ M **2** elicited a significantly higher (ANOVA, $p < 0.01$) reporter response in the presence of ER α and ER β than either receptor alone (Figure 2B). By contrast, ER β had no effect on the marginal ER α -mediated response at 30 nM **2**, implying that an activation of either ER that could provide for a sizeable pool of heterodimers is likely necessary before positive modulation by **2** becomes apparent. Interestingly, the presence of ER β dampened the ER α -mediated response to 1 nM estradiol, indicating a lower transcriptional activation of the tk promoter by both ER as compared to ER α alone in the presence of

Table 1. Comparison of Relative Binding Affinities and Transcriptional Activities of Deoxybenzoins 1–11 to Those of Daidzein and Estradiol

Compound	RBA α^a	RBA β^a	RBS β^b	TA α^c	TA β^c
Estradiol	100	100		5.30	5.81
1	0.0209 \pm 0.0018	0.0157 \pm 0.0016	0.75	2.89	5.59
2	0.0506 \pm 0.0036	0.2734 \pm 0.0264	5.40	6.08	9.15
3	0.0189 \pm 0.0032	0.0075 \pm 0.0004	0.40	ND	ND
4	0.0522 \pm 0.0046	0.0280 \pm 0.0027	0.54	1.59	3.22
5	0.0220 \pm 0.0027	0.0276 \pm 0.0022	1.25	2.82	5.29
6	0.0367 \pm 0.0029	0.0517 \pm 0.0040	1.41	2.05	2.91
7	0.0109 \pm 0.0016	0.0107 \pm 0.0011	0.98	3.31	6.66
8	0.0697 \pm 0.0051	0.0341 \pm 0.0042	0.49	1.37	1.43
9	0.0087 \pm 0.0014	0.0033 \pm 0.0002	0.38	1.63	3.05
10	0.0022 \pm 0.0003	0.0036 \pm 0.0001	1.64	ND	ND
11	0.0556 \pm 0.0085	0.0906 \pm 0.0085	1.63	2.45	5.04
Daidzein	0.0309 \pm 0.0029	0.0199 \pm 0.0014	0.64	7.60	10.47

ND, not determined.

^a RBA values for ER α (RBA α) and ER β (RBA β) are mean \pm SEM of at least three independent experiments, with those of 17 β -estradiol arbitrarily set equal to 100.

^b Relative ER β binding selectivity (RBS β) is calculated by [RBA β]/[RBA α].

^c Transcriptional activities in the presence of ER α (TA α) or ER β (TA β) are calculated by the following: [reporter response in the presence of ER and 10 μ M test compound]/[reporter response in the presence of ER and in the absence of test compound]. The TA α and TA β values of 1 nM 17 β -estradiol are given for comparison.

the hormone. Notably, the experiment of Figure 2C shows that normalizing deoxybenzoin responses to the respective vehicle responses rather than to the activity of reporter alone also results in a higher response with both receptors than with either receptor alone, excluding that changes in ER constitutive activity might account for the enhancement of deoxybenzoin activation of the response upon coexpression of ER α and ER β . Figure 2C shows, in addition, that changes in ER constitutive activity(ies) might not account for the decreasing of the estradiol response either, further suggesting that ER heterodimerization is responsible for the opposite modulator effects of estradiol and 2. With several deoxybenzoins exhibiting an ER β binding selectivity and a bias toward ER β -mediated transcription comparable to 2 (Table 1), the inference is that they too could positively modulate ER transcriptional activity via ER β .

Stimulation of Cell Proliferation

Stimulation of proliferation of breast cancer cells by estrogens is believed to depend primarily on ER α [18]. In light of this, deoxybenzoin stimulation of the proliferation of MCF-7 breast cancer cells, known to express much higher amounts of ER α than ER β , was compared to that of MDA-MB-231 breast cancer cells, which are ER α negative [19, 20]. Estradiol stimulation of MCF-7 cell proliferation attained maximum value (288% that of hormone-free cells) by 0.1 nM hormone, in accordance with reports that the complement of estradiol binding sites of MCF-7 cells is fully occupied by hormone concentrations \geq 0.1 nM [21]. We found that IC₅₀ values for 50% inhibition of 0.3 nM [³H]estradiol binding to MCF-7 cells by increasing concentrations of radioinert estradiol, 2, and daidzein are 0.41 \pm 0.04, 405 \pm 59, and 1717 \pm 177 nM, respectively, matching RBA α values as determined using FP. We also found that whole-cell hormone binding is fully competed at 0.003, 3, and 10 μ M, respectively (Figure 3A), suggesting that 0.1 and 1 μ M of 2 can effectively compete (\geq 50%) with postmenopausal

levels of estrogen for binding to the native ER β and ER α , respectively. Thus, the use of 2 for hormone replacement therapy (HRT) may be envisaged.

In the absence of hormone, 0.1–10 μ M 2 stimulated the proliferation of MCF-7 cells significantly, but 100 μ M 2 inhibited proliferation (Figure 3B), in accordance with reports that high concentrations of phytoestrogens impact ER-independent growth-inhibitory effects [22]. By contrast, the proliferation of MDA-MB-231 cells in the presence of 10 μ M 2 was not significantly higher than basal proliferation in its absence, implying that deoxybenzoin stimulation of MCF-7 cell proliferation likely depends on ER α . All deoxybenzoins but 10 and 11 exhibited a similar profile of proliferative response. Table 2 shows that the effective concentration (EC₂₅) of deoxybenzoin stimulation of proliferation to a level equal to 25% that of 0.1 nM estradiol varied from 0.02 to $>$ 10 μ M (column 2), and that the efficacy of the response at 10 μ M deoxybenzoin was high to moderate (i.e., \geq of 25% that of 0.1 nM estradiol) with 1–4 and 7–9 and weak (i.e., 10%–25% that of 0.1 nM estradiol) with 5 and 6, whereas 10 and 11 inhibited rather than stimulated proliferation (column 4). Inspection of the structures of Figure 1 reveals that stimulation of proliferation is associated with R₄ = OH, whether derivatized or not. We wondered whether RBA α or RBA β of 1–4 and 7–9 correlated with their proliferative potencies. While 2, 4, and 8 have RBA α values of \sim 0.05, 2 binds ER β with \sim 10-fold higher affinity and is \sim 300 times more potent than either 4 or 8, indicating that ER β binding of 2 likely enhances its proliferative potency. Similarly, 1 and 3 have RBA α values of \sim 0.02, but 1 binds ER β with \sim 2-fold higher affinity and is \sim 5 times more potent than 3. A similar comparison of 7 and 9 further supports the notion of a dependence of the proliferative potency of deoxybenzoins on their binding of ER β . Significantly, it has been reported that estrogen stimulation of MCF-7 cell proliferation involves activation of src kinase as well as transcription of cell cycle genes by ER β and ER α [23–26].

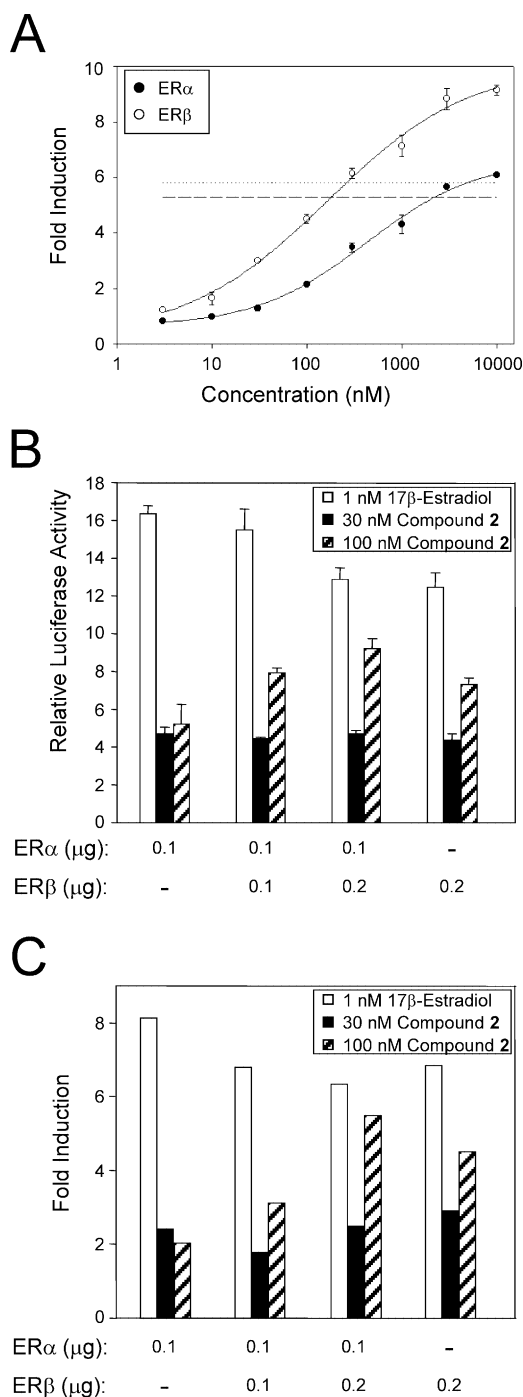


Figure 2. Deoxybenzoin Regulation of ER α - and/or ER β -Mediated Gene Expression

(A) Induction by 2 of ER α and ER β transcriptional activity. HEK-293 cells were transfected with an ERE-luciferase reporter and plasmids expressing human ER α (solid circles) or ER β (open circles) and incubated with vehicle, estradiol, or increasing concentrations of 2. Normalized levels of luciferase activity were expressed relative to those of transfected cells treated only with vehicle (set to 1). The levels of luciferase activity induced by 1 nM estradiol in cells expressing ER α (dashed line) or ER β (dotted line) are shown for comparison.

(B and C) Coexpression of ER α and ER β enhances the transcriptional efficacy of 2. HEK-293 cells were transfected with the ERE-

Activation of Reporter Gene Expression

To elaborate on the transcription activation potencies and efficacies of the synthesized deoxybenzoin, we used MCF-7 cells (clone D5L) stably transfected with an ERE-dependent luciferase reporter endowed with a strong (β -globin) promoter [27]. Estradiol stimulation of reporter expression attained maximum level (351 % that of hormone-free cells) by 0.1 nM hormone. Table 2 shows that the effective concentration (EC_{50}) of deoxybenzoin stimulation of luciferase expression to a level equal to 50% that of 0.1 nM estradiol varied from 0.06 to >10 μ M (column 5) and that the efficacy of the response at 10 μ M deoxybenzoin was high to moderate with 1, 2, 5–8, and 11 and weak with 3, 4, 9, and 10 (Table 2, column 7 and Figure 3C). It is currently unclear whether the ~ 10 -fold higher EC_{50} for activation of ER α -mediated reporter response to 2 in HEK-293 cells (Figure 2A) as compared to that in MCF-7 cells reflects modulation of ER α activity by ER β rather than the inherent difficulty of transfected ER to replicate hormonal events as staged by endogenous ER. The high constitutive activity of transfected ER points to the latter alternative as being more likely. By comparison, 1, 2, 7, and 8 stimulate gene expression as well as MCF-7 cell proliferation; 3, 4, and 9 stimulate proliferation more than gene expression; 5, 6, and 11 stimulate gene expression more than proliferation; and 10 stimulates neither. Thus, unlike cell proliferation, F, NO₂, or H substitution for 4'-OH is not detrimental to gene expression, indicative of different determinants guiding deoxybenzoin stimulation of these responses. Notably, the correlation between induction efficacies at 10 μ M deoxybenzoin and the TA α values of Table 1 ($r = 0.812$, $p = 0.008$) gets stronger when 2 is excluded ($r = 0.889$, $p = 0.003$), and the trend is maintained when the second most ER β -selective and transcriptionally biased deoxybenzoin (11) is excluded also ($r = 0.901$, $p = 0.006$), which may be taken to suggest that ER β binding selectivity and transcriptional bias of 2 and 11 interfere with ER α being the sole determinant of the gene expression response in their presence.

ER-mediated activation of transcription from ERE-dependent promoters depends on two transcription activation functions (AF), AF1 and AF2 [3]. While the AF2 of ER α and ER β are of similarly high strength, AF1 of ER α is much more potent than ER β , resulting in a higher activation of transcription from ERE-dependent promoters [26]. We have shown above that the ER-dependent reporter response to 2 is positively modulated by ER β ; the opposite is true for the response to estradiol. Pettersson et al. [28] have reported that ER β exercised a similar modulation of their reporter responses to estradiol and genistein, a phytoestrogen of much higher selectivity for ER β (RBA β = 43.9, RBS β = 51) than 2. Since ER α and ER β colocalize in nuclear clusters housing chromatin remodeling components [29], they could form

luciferase reporter and plasmids expressing human ER α and/or ER β and incubated with estradiol or 2, as indicated. Normalized levels of luciferase activity were expressed relative to those of cells transfected with the reporter alone and treated with vehicle (set to 1, [B]) or of transfected cells treated only with vehicle (set to 1, [C]).

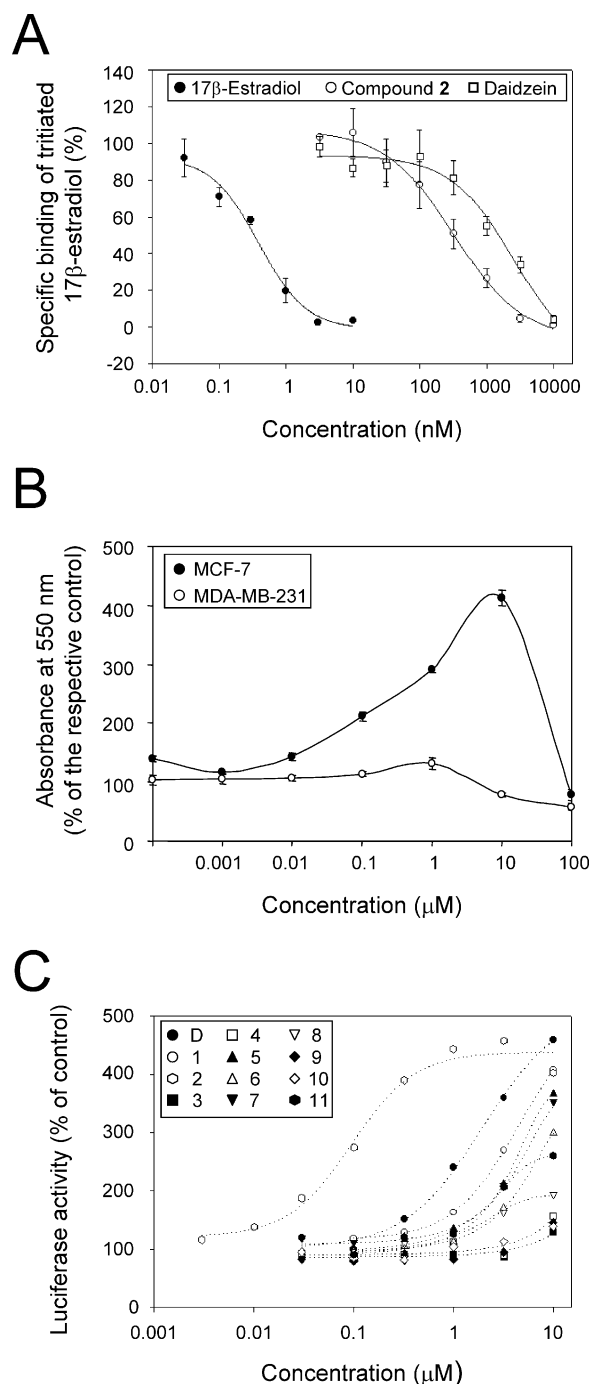


Figure 3. Biological Responses versus Deoxybenzoins Binding of Native ER

(A) Displacement of tritiated estradiol from the native ER of intact MCF-7 cells by increasing concentrations of radioinert estradiol, 2, and daidzein, as indicated

(B) Percent stimulation of the basal (estrogen-free) proliferation of MCF-7 (ER α -positive) and MDA-MB-231 (ER α -negative) cells in the presence of increasing concentrations of 2, as obtained using the MTT assay.

(C) Induction of luciferase expression in MCF-7:D5L cells using increasing concentrations of 1-11, as indicated.

transcriptionally active heterodimers. These could sensitize the AF-1-mediated activation of the gene expression program of MCF-7 cells to the presence of ER β -selective and transcriptionally biased deoxybenzoins.

Induction of Alkaline Phosphatase Activity

To ensure that activity is not restricted to breast cancer cells, we tested deoxybenzoins estrogenicities using Ishikawa endometrial adenocarcinoma cells. It has been reported that hormonal induction of alkaline phosphatase (AlkP) activity in these cells is estrogen specific, with androgens, progestins, glucocorticoids, and mineralocorticoids exhibiting negligible activity [30], and that induction by phytoestrogens is an indicator of the intrinsic estrogenic activity of these compounds [31]. As already observed with the other responses, estradiol stimulation of AlkP expression attained maximum level (414% that of hormone-free cells) by 0.1 nM hormone. Table 2 shows that the effective concentration (EC₂₅) of deoxybenzoins stimulation of AlkP expression to a level equal to 25% that of 0.1 nM estradiol varied from 0.06 to >10 μ M (column 8), and that the efficacy of the response at 10 μ M deoxybenzoins was high to moderate with 1, 2, 5, 7, and 11 and weak or marginal with 3, 4, 6, and 8-10 (column 10). In fact, 1, 2 and 7 stimulate AlkP expression as well as MCF-7 cell proliferation; 3, 4, 8, and 9 stimulate proliferation more than AlkP expression; 5 and 11 stimulate AlkP expression but either fail to stimulate proliferation (5) or inhibit it (11); and 6 and 10 stimulate neither. Thus, deoxybenzoins that potently transactivate the luciferase reporter [rank order of potencies (2 > 1 \geq 5 \geq 7 \geq 6 \geq 11)] stimulate AlkP expression as well (2 > 5 \geq 7 \geq 1 \geq 11). Yet, 5 and 11 cannot stimulate the proliferation of breast cancer cells, which makes them a potentially safe choice for HRT.

We next looked for structural features that could account for the differences in activity described above. Whether gene-transactivation or cell-proliferation wise, substitutions in positions R1 and R2 lead to a decrease in both RBA β and estrogenic activity (compare 7 with R1 = Cl and 4 with R2 = OH to 2 with R1 = H and R2 = H). The presence of 4'-OH is associated with higher proliferative activity as well as RBA (compare 5 with R4 = F to 2 with R4 = OH). However, derivatization of 4'-OH lowers both RBA and estrogenic activity (compare 1 to 2, 3 to 4, and 7 to 9), and this is also the case following exchange of 4'-OH for larger substitutes (compare 10 and 6 to 2). In fact, 10 seems to be totally inactive in all three assays (Table 2). Interestingly, substitution of F (but not Br) for 4'-OH produced a deoxybenzoins (5) relatively active with respect to gene expression but proliferatively inert, whereas presence of 3'-Br in conjunction with lack of 4'-OH produced a deoxybenzoins (11) somewhat less potent than 5 gene-expression wise, yet clearly inhibitory cell-proliferation wise.

Models of Deoxybenzoins Binding of ER β

Conformational Analysis

Hormonal activity of steroidal and nonsteroidal estrogens is associated with H bonding ability of hydroxyl groups mimicking the 3-OH and the 17 β -OH of estradiol, an O-O distance between these groups in the range

Table 2. Biological Responses to the Deoxybenzoins 1–11, Daidzein, and Estradiol

Compound	MCF-7 Proliferation			Reporter Gene Expression			Alkaline Phosphatase Activity		
	EC ₂₅ ^a (μM)	Relative Potency ^b	Efficacy ^c	EC ₂₅ ^a (μM)	Relative Potency ^b	Efficacy ^c	EC ₂₅ ^a (μM)	Relative Potency ^b	Efficacy ^c
1	0.91 ± 0.05	0.0007	197 ± 7	3.42 ± 0.17	0.0008	407 ± 47	3.83 ± 0.71	0.0005	179 ± 16
2	0.02 ± 0.01	0.0399	413 ± 14	0.06 ± 0.01	0.0461	443 ± 49	0.06 ± 0.01	0.0331	319 ± 51
3	4.96 ± 1.40	0.0001	178 ± 4	>10	<0.0003	130 ± 23	>10	<0.0002	116 ± 17
4	4.94 ± 1.39	0.0001	186 ± 7	>10	<0.0003	157 ± 12	>10	<0.0002	141 ± 17
5	>10	<0.0001	129 ± 3	3.62 ± 1.16	0.0008	367 ± 60	2.13 ± 0.86	0.0009	225 ± 16
6	>10	<0.0001	123 ± 11	8.54 ± 0.86	0.0003	298 ± 37	8.25 ± 1.75	0.0002	157 ± 10
7	1.06 ± 0.47	0.0006	258 ± 5	5.33 ± 1.05	0.0005	353 ± 46	2.46 ± 0.22	0.0008	249 ± 18
8	6.75 ± 2.40	0.0001	166 ± 10	>10	<0.0003	194 ± 9	>10	<0.0002	116 ± 8
9	6.19 ± 3.14	0.0001	170 ± 12	>10	<0.0003	146 ± 32	>10	<0.0002	132 ± 6
10	NA		95 ± 7	>10	<0.0003	139 ± 58	7.51 ± 0.95	0.0002	104 ± 12
11	NA		75 ± 9	9.52 ± 0.39	0.0003	260 ± 45	5.32 ± 1.80	0.0004	180 ± 18
Daidzein	0.04 ± 0.03	0.0157	346 ± 13	0.75 ± 0.15	0.0037	459 ± 17	0.33 ± 0.10	0.0058	290 ± 25
Estradiol	6.40* ± 4.39	100	288 ± 62	27.67* ± 9.24	100	351 ± 14	18.77* ± 1.99	100	414 ± 67

NA, not applicable; *, pM.

^aEC₂₅ and EC₅₀ values are test compound concentrations required to achieve 25% and 50%, respectively, of the effect of 0.1 nM estradiol. Values are mean ± SEM of at least three independent experiments.^bRelative potency was calculated by the following: $[100 \times \text{EC}_{25} \text{ (or EC}_{50}) \text{ of estradiol}] / [\text{EC}_{25} \text{ (or EC}_{50}) \text{ of test compound}]$.^cAll compounds were tested at 10 μM. Estradiol was tested at 0.1 nM. Efficacies (% of control, mean ± SEM of three independent experiments) indicate absorbance of MTT-formazan in MCF-7 cells (cell proliferation), luciferase activity in MCF-7:D5L cells (reporter gene expression), and alkaline phosphatase activity in Ishikawa cells.

10.9–12.5 Å, and hydrophobic centers mimicking steric 7 α and 11 β substituents. Conformational analysis of 1–11 using molecular mechanics calculations [32] revealed that each can adopt three low energy conformations stabilized by the formation of a hydrogen bond between 2-OH and the carbonyl oxygen (Figure 4A). Conformers I and III are bended and further stabilized through the hydrophobic interaction of the phenyl rings, while conformer II, with a relative energy of ~0.5 kcal/mol compared to the global minimum conformer I, exhibits an extended shape comparable to daidzein and genistein. Conformer II of 1–4 and 7–9 has a O–O distance between the 4 and 4'-OH groups of 12.0 Å, in agreement with one of the criteria for optimum interaction with ER.

Alignment

To deduce structure-activity relationships, docking calculations were performed and followed by correlation between the experimental RBA values and the calculated binding energy E_{bind} . Since the initial orientation of the ligand inside the binding pocket is critical, preliminary calculations were performed to deduce the initial alignments using the published crystal structure of genistein bound to the LBD of ER β [8]. In this structure, 4'-OH interacts with the side chains of Glu305, Arg346, and a buried water molecule mimicking the A-ring of estradiol, whereas the 7-OH group of the flavone core is H bonded to His475 at the distal end of the binding cavity (see Figure 5A). In addition, the keto- and 5-OH groups are buried toward the α -face pocket (roughly the 7 α position of estradiol). Deoxybenzoins 1, 3, 5, 6, and 9–11 (lacking a 4'-OH) were positioned in the ER β -genistein structure fitting the phenyl ring of genistein with 4-OH. Energy minimization calculations were performed by rotating the molecule around the O–O axis 180° inside the binding pocket. In all cases, in the minimum energy structure the keto- and the 2-OH groups were oriented to the α -face of the cavity. Since 2, 4, 7, and 8 bear two hydroxyl groups that could play a role

analogous to the OH of the A-ring of estradiol, geometry optimizations were performed, allowing 4-OH to interact with Glu305 and Arg346, the 4'-OH to interact with His475 and vice versa, and rotating around the O–O

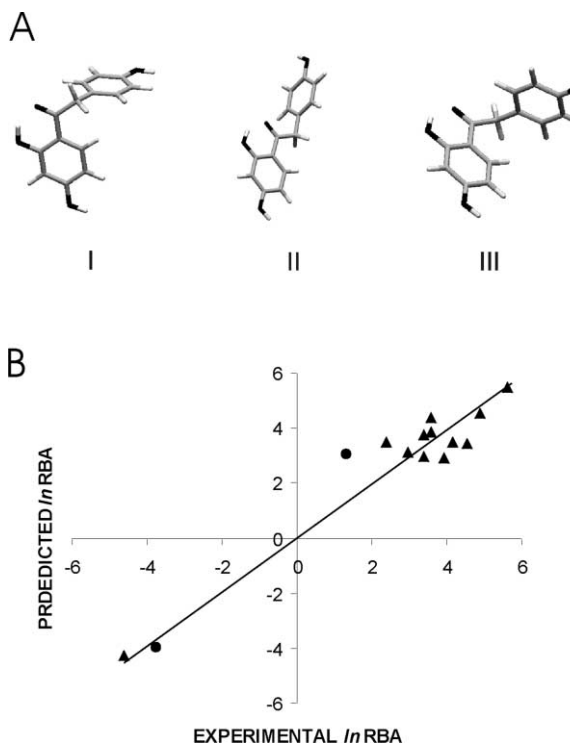


Figure 4. Low-Energy Conformers of Deoxybenzoins 2 and ER Binding Affinity Predictions

(A) The three low energy conformers of 2 as deduced using Monte Carlo MM2 conformational search.

(B) Plot of experimental lnRBAs versus predicted lnRBAs. Triangles represent the training set, and circles represent the test set.

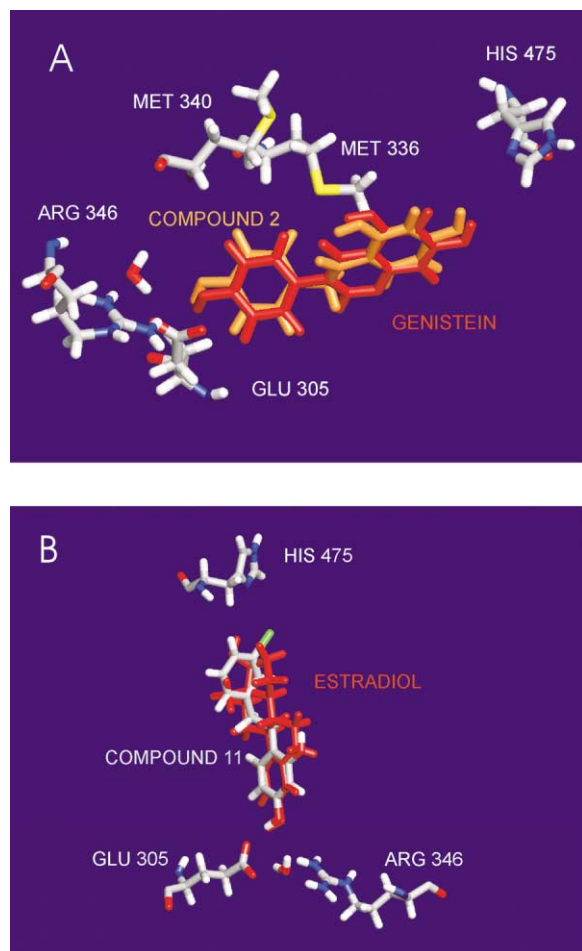


Figure 5. Comparative Molecular Modeling of Deoxybenzoin Binding of ER β

(A) Superposition of Genistein and 2 in the ER binding site as deduced using PrGen calculations. Met336 and Met340 lie above and very close to keto and OH groups of the ligand.

(B) Superposition of estradiol and 11 in the ER binding site. The position Br atom of 11 is close to the position of 16 α - substituent of estradiol.

axis 180°. The minimum energy structures thus obtained were subjected to docking calculations which revealed that 2, 7, and 8 prefer to bind similar to genistein, with the keto- and 2-OH groups in similar positions to the corresponding groups of genistein, while 4 adopts a similar position to 1, 3, 5, 6, and 9–11.

Scoring

To produce a model relating calculated energy terms to binding affinities, the molecules were aligned as above, positioned in the ER β cavity, and subjected to docking-scoring iterative calculations using PrGen 2.0 software. Theoretical binding affinities are estimated by evaluating ligand-receptor interaction energies, ligand desolvation energies, and changes in both ligand-internal energy and ligand-internal entropy upon receptor binding: $E_{\text{binding}} \approx E_{\text{ligand-receptor}} - T\Delta S_{\text{binding}} - \Delta G_{\text{solvation,ligand}} + \Delta E_{\text{internal,ligand}}$ [33]. Binding affinities are then obtained by linear regression between $\ln RBA$ and E_{binding} . A training set of 12 molecules was used, including estradiol and daidzein, while keep-

ing genistein and compound 2 for the test set. The deduced correlation between experimental and predicted $\ln RBA$ (Figure 4B) has a total correlation coefficient of 0.932 (r^2) with an RMS prediction error of 0.68. Genistein and 2 were then positioned within the fixed receptor cavity and "Monte Carlo minimized" to yield a satisfactory correlation between experimental and theoretical values for the test molecules (Figure 4B), suggesting that the model is apt to predict relatively high and low RBA values.

According to the deduced model, genistein, daidzein, 2, 7, and 8 are similarly positioned, and 2, in particular, superimposes to genistein perfectly (Figure 5A). Daidzein also superimposes to genistein, but the lack of 5-OH seems to hamper its ER β binding selectivity. As already reported for genistein [8], the ER β binding selectivity of 2 could be due to an interaction with Met336 (Leu384 in ER α), one of the two cavity-lining residues that differ between ER α and ER β . Met336 and Met340 interact through a hydrogen bond between backbone NH and CO, and both lie very close above the keto and the 2-OH groups of 2 (Figure 5A). The sulfur atom of both residues, bearing a weak polar character, could interact with these two groups, thus contributing to the selectivity toward ER β . While 7 and 8 also bind like genistein, they bear a Cl atom toward a hydrophobic pocket very close to Val484, the first residue of helix 12, which possibly explains their lower activity compared to 2. Deoxybenzoins 1, 3–6, and 9–11 are oriented in the cavity in the opposite direction compared to genistein, with the two aromatic rings perpendicular to each other, thus deviating from the planar shape of genistein (Figure 5B). In this orientation, the 3'-Br atom of 11 is found in the same position like 16 α -H of estradiol. In estradiol, Br substitution for 16 α H is very well tolerated (similar RBA) [35], which is in accordance with the relatively high RBA β of 11.

In light of the above, it appears that a deoxybenzoin orientation that provides for H bonding of His475 (His 524 in ER α) with 4-OH is necessary for activation of cell proliferation as well as gene expression to a level comparable or even higher than that of estradiol (e.g., 2); that derivatization of 4'-OH is more detrimental to stimulation of cell proliferation than gene expression (e.g., 1); and that deoxybenzoins lacking a 4' O atom but possessing appropriate 3' substituents (5, 11) may inhibit or fail to promote cell proliferation while maintaining most of the efficacy of 1 and 2 to activate gene expression, albeit with reduced potency. On the whole, it appears that the proliferative activity of 1–4 and 7–9 should be correlated with the presence of two O atoms being able to meet one of the structural requirements for estrogenicity. Since 1 and 2 bear two OH atoms at a distance of 12 Å and the 4'-OH of 1 is methylated, they likely contact His475 as H-bond acceptor and donor, respectively. It has been reported, however, that ligand H bonding of an acceptor His475 begins a H-bond network that involves Glu371, extends to the C terminal of helix 11, and stabilizes the agonist position of helix 12; and that disruption of this network lowers the gene expression potency and efficacy of ER [34]. However, our data suggest that it is deoxybenzoin activation of cell proliferation rather than gene expression that is primarily affected by disruption of the H-bond network, and that

one can synthesize ER β -selective antiproliferative deoxybenzoin lacking 4'-OH, which nevertheless can effectively activate gene expression (e.g., 11). Van der Waals contacts of appropriate 3' substituents (e.g., Br) with Ile373 (Met421 in ER α) [8], the other cavity-lining molecule that differs between ER α and ER β , may be instrumental for the maintenance of gene expression activity of 11. Thus, it appears that certain deoxybenzoin can replicate the activity of established selective ER modulators (SERMs) such as tamoxifen, and subtle structural alterations of the deoxybenzoin scaffold can give rise to receptor-ligand complexes of such a topology and interaction with coregulators that could provide for the dissociation of the proliferative and gene expression responses of breast cancer cells to estradiol [36].

Significance

While HRT of postmenopausal syndromes is associated with a higher risk of developing breast and/or endometrial cancer, consumption of dietary estrogens is thought to have the opposite effect [1, 2]. However, the exact mechanisms underlying the complex estrogenic/antiestrogenic behavior of most phytoestrogens remain a matter of conjecture. It is believed that the beneficial effects associated with consumption of phytoestrogens reflect their preferential activation of ER β function(s) [5]. The present report shows that 0.1–3 μ M 2 can effectively compete with postmenopausal estrogen for binding to the native ER and that the ER β binding selectivity and transcriptional bias of 2 is accompanied by higher activation of transcription of estrogen target genes compared to estradiol, which makes 2 potentially useful for HRT. It is known, however, that the proliferation of breast cancer cells is driven predominantly by ER α , the number of ER α -positive proliferating cells of the human breast increases with advancing age [37], and that, while the level of ER β decreases in preinvasive mammary tumors, that of ER α increases instead [38]. In this light, a HRT based on 2 could increase breast cancer risk. Interestingly, from the subset of ER β -selective and transcriptionally competent deoxybenzoin lacking a 4'-OH, one (11) can activate gene expression and inhibit cell proliferation at the same time. Such deoxybenzoin are a potentially safe choice for HRT, especially if their RBA β were to improve while their selectivity and transcriptional bias toward ER β is maintained compared to 11.

Experimental Procedures

NMR spectra were recorded on Bruker DRX 400 and Bruker AC 200 spectrometers [1 H (400 and 200 MHz) and 13 C (50 MHz)]; chemical shifts are expressed in ppm downfield from TMS. The 1 H- 13 C and the 1 H- 13 C NMR experiments were performed using standard Bruker microprograms.

Preparation of Deoxybenzoin

A phenol (0.050 mol) and an arylacetic acid (0.050 mol) were dissolved into freshly distilled BF $_3$ ·Et $_2$ O under argon. The mixture was stirred at 80°C and then poured in an ice bath. The resulting mixture was extracted with ethyl acetate, and the organic layer was washed with aq. NaHCO $_3$, dried (Na $_2$ SO $_4$), and evaporated. The residue was purified by column chromatography on silica gel, using a mixture of dichloromethane and methanol. All compounds were identified

by NMR (1 H-NMR, 13 C-NMR, HMBC, HMQC, COSY-LR) and MS-Cl spectroscopy.

2,4-Dihydroxy-4'-Nitrodeoxybenzoin [2-(4-Nitrophenyl)-1-(2,4-Dihydroxyphenyl)Ethanone] 6

1 H NMR (MeOD, 400 MHz) δ : 8.17 (2H, d, J = 8.0 Hz, H-3'/H-5'), 7.85 (1H, d, J = 8 Hz, H-6), 7.51 (2H, d, J = 8.0 Hz, H-2'/H-6'), 6.38 (1H, dd, J = 8.0/1.5 Hz, H-5), 6.27 (1H, d, J = 1.5 Hz, H-3), 4.42 (2H, s, CH $_2$). 13 C NMR (MeOD, 50 MHz) δ : 202.5 (CO), 166.8 (C-4/C-2), 148.5 (C-4'), 144.2 (C-1'), 134.6 (C-6), 132.4 (C-2'/C-6'), 124.2 (C-3'/C-5'), 114.0 (C-1), 110.0 (C-5), 104.2 (C-3), 46.8 (CH $_2$). MS-Cl, m/z 274 (M+H) $^+$.

5-Chloro-2,3,4,4'-Tetrahydroxy-Deoxybenzoin [1-(5-Chloro-2,4-Dihydroxyphenyl)-2-(3,4-Dihydroxyphenyl)Ethanone] 8

1 H NMR (Me $_2$ CO-d $_6$, 400 MHz) δ : 7.82, (1H, s, H-6), 6.73 (1H, d, J = 8.0 Hz, H-5'), 6.73 (1H, d, J = 1.5 Hz H-2'), 6.60 (2H, dd, J = 8.0/1.5 Hz, H-6'), 6.40 (1H, s, H-3), 4.02 (2H, s, CH $_2$). 13 C NMR (CDCl $_3$, 50 MHz) δ : 204.1 (CO), 165.9 (C-4), 162.3 (C-2), 148.2 (C-3'), 146.7 (C-4'), 134.3 (C-6), 128.2.0 (C-1'), 123.7 (C-6'), 118.3 (C-2'), 118.3 (C-5'), 114.5 (C-5), 114.5 (C-1), 105.7 (C-3), 47.1 (CH $_2$). MS-Cl, m/z 295 (M+H) $^+$.

5-Chloro-2,4-Dihydroxy-4'-Ethoxydeoxybenzoin [1-(5-Chloro-2,4-Dihydroxyphenyl)-2-(4-Ethoxyphenyl)Ethanone] 9

1 H NMR (Me $_2$ CO-d $_6$, 400 MHz) δ : 7.82, (1H, s, H-6), 7.06 (2H, d, J = 8.0 Hz, H-2'/H-6'), 6.78 (2H, d, J = 8.0 Hz, H-3'/H-5'), 6.38 (1H, s, H-3), 4.02 (2H, s, CH $_2$), 3.91 (2H, q, CH $_2$ CH $_3$), 1.32 (3H, t, CH $_3$). 13 C NMR (CDCl $_3$, 50 MHz) δ : 203.9 (CO), 164.0 (C-4), 162.1 (C-2), 159.9 (C-4'), 134.3 (C-6), 132.0 (C-2'/C-6'), 127.9 (C-1'), 116.3 (C-3'/C-5'), 113.9 (C-1), 113.9 (C-5), 105.2 (C-3), 64.3 (CH $_2$ CH $_3$), 44.9 (CH $_2$), 15.8 (CH $_3$ CH $_3$). MS-Cl, m/z 307 (M+H) $^+$.

4'-Bromo-2,4-Dihydroxydeoxybenzoin [2-(4-Bromophenyl)-1-(2,4-Dihydroxyphenyl)Ethanone] 10

1 H NMR (MeOD, 400 MHz) δ : 7.79 (1H, d, J = 8 Hz, H-6), 7.42 (2H, d, J = 8.0 Hz, H-3'/H-5'), 7.14 (2H, d, J = 8.0 Hz, H-2'/H-6'), 6.37 (1H, dd, J = 8.0/1.5 Hz, H-5), 6.27 (1H, d, J = 1.5 Hz, H-3), 4.13 (2H, s, CH $_2$). 13 C NMR (MeOD, 50 MHz) δ : 203.7 (CO), 167.0 (C-4/C-2), 135.8 (C-1'), 134.6 (C-6), 133.0 (C-2'/C-6'), 133.0 (C-3'/C-5'), 121.7 (C-4'), 113.9 (C-1), 108.9 (C-5), 104.3 (C-3), 45.7 (CH $_2$). MS-Cl, m/z 308 (M+H) $^+$.

3'-Bromo-2,4-Dihydroxydeoxybenzoin [2-(3-Bromophenyl)-1-(2,4-Dihydroxyphenyl)Ethanone] 11

1 H NMR (MeOD, 400 MHz) δ : 7.79 (1H, d, J = 8 Hz, H-6), 7.47 (1H, t, J = 1.5 Hz, H-2'), 7.40 (2H, dt, 7.14 J = 8.0/1.5 Hz, H-4'), 7.27–7.16 (2H, m, H-5'/H-6'), 6.37 (1H, dd, J = 8.0/1.5 Hz, H-5), 6.27 (1H, d, J = 1.5 Hz, H-3), 4.13 (2H, s, CH $_2$). 13 C NMR (MeOD, 50 MHz) δ : 210.6 (CO), 167.3 (C-4), 167.2 (C-2), 139.5 (C-1'), 134.7 (C-6), 134.1 (C-2'), 131.8 (C-4'), 131.8 (C-4'), 131.4 (C-6'), 130.0 (C-5'), 113.9 (C-1), 109.8 (C-5), 104.2 (C-3), 45.3 (CH $_2$). MS-Cl, m/z 308 (M+H) $^+$.

Daidzein, 7-Hydroxy-3-(4-Hydroxyphenyl)-Chromen-4-One

In a solution of 2 (2 mmol) in DMF (6 ml), freshly distilled BF $_3$ ·Et $_2$ O (6.3 ml) was added under argon. The mixture was heated at 50°C, and a solution of methanesulphonyl chloride (1 ml) in dry DMF (1.5 ml) was added slowly. After reaction at 80°C for 1 hr, the mixture was cooled to room temperature and poured into a large volume of ice-cold aq. sodium acetate (12 g/100 ml), then extracted with ethyl acetate, and the organic layer was dried and concentrated. The residue was purified by column chromatography on silica gel using a mixture of dichloromethane and methanol. For 1 H NMR, see [39].

ER Binding Affinities

These were determined using a fluorescent ER ligand (ES2) and recombinant ER α or ER β with a Beacon 2000 Fluorescence Polarization (FP) System (PanVera) as described by Bolger et al. [17]. Test compound competition of 1 nM ES2 binding to ER obeys the following scheme: Test + ER-ES2 (high FP) \rightarrow ER-Test + ES2 (low FP). IC $_{50}$ values for estradiol thus determined using ER α and ER β were 1.24 \pm 0.07 and 1.45 \pm 0.18 nM, respectively. Deoxybenzoin competition of ES2 binding to ER gave IC $_{50}$ estimates from which RBA values were calculated by [(IC $_{50}$ estradiol/IC $_{50}$ competitor) \times 100]. FP values for ER-bound ES2 in the absence of competitor and of both ER and competitor served as negative and positive displacement controls, respectively. ES2 displacements were curve fitted using SigmaPlot 4.0 (SPSS Inc).

Stimulation of Cell Proliferation

MCF-7 and MDA-MB-231 human mammary adenocarcinoma cells (ATCC, Rockville, MD) were cultured at 37°C in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (Biocrom KG, Berlin) in 5% CO₂ and subcultured using a trypsin 0.25%–EDTA 0.02% solution. To assess cell proliferation, a modification of the MTT assay was used [40]. Briefly, cells were plated in 96-well flat-bottomed microplates at a density of 10,000 cells/well in phenol-red-free medium supplemented with 1% dextran-coated-charcoal-pretreated FBS (DCC-FBS) [41]. Twenty-four hours later, serial dilutions of test compounds were added (initial dilution in DMSO, further dilutions in culture medium), fresh media with test compounds was added every 48 hr, and after 6 days the medium was removed and the cells were incubated with 1 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, St. Louis, MO) in serum-free, phenol-red-free medium for 4 hr. The MTT-formazan produced was solubilized in isopropanol, and absorbance at 550 nm versus 690 nm was measured. Cells that received only medium served as baseline controls, while those treated with estradiol (Sigma) served as positive controls. The difference in the responses of MCF-7 and MDA-MB-231 cells was taken to measure estrogenic activity.

Transactivation of Reporter Gene Expression

HEK-293 cells were maintained and transfected as described by Pettersson et al. [28], with minor modifications. In brief, cells were seeded in 6 cm dishes and transfected with the calcium phosphate coprecipitation method using 1.5 µg of pERE-tk-Luc reporter plasmid, 1 µg of internal control plasmid pCMX-Gal, 0.1 µg of pSG5-hER α or pSG5-hER β expression plasmids alone, or 0.1 µg of pSG5-hER α together with 0.1 or 0.2 µg of pSG5-hER β , as indicated. Sixteen to eighteen hours after transfection, cells were washed with phosphate buffered saline (PBS) and incubated in fresh medium with or without 1 nM estradiol, the indicated deoxybenzoins concentrations, or vehicle (0.1% DMSO) for 24 hr. Luciferase activities were normalized relative to β -galactosidase expression levels. Plasmids pERE-tk-Luc, pSG5-hER α , and pSG5-hER β have been described [26].

MCF-7 cells were transfected with the luciferase reporter plasmid ERE- β Glob-Luc [27] and neomycin resistance plasmid pWL2neo (Stratagene) using standard methodology. Transfectants were selected using geneticin (Stratagene). The geneticin-resistant clone D5L of MCF-7 cells, selected for its prominent induction of reporter expression by estradiol and low basal expression in its absence, as assessed using the Steady-Glo Luciferase Assay System (Promega), was cultured as described above for MCF-7 cells. To assess luciferase induction by deoxybenzoins, MCF-7:D5L cells were plated in 96-well flat-bottomed microplates at a density of 10,000 cells/well in phenol-red-free MEM supplemented with 5% DCC-FBS; 72 hr later, fresh medium was added to the cells followed by test compounds (initial dilution in DMSO, further dilutions in culture medium). Luciferase activity was assayed 16 hr later using a Galaxy 1258 plate reader (BioOrbit). Cells that received only medium served as baseline expression controls.

Whole-Cell ER Binding Assay

The assay was carried out as described by Lascombe et al. [21], with minor modifications. MCF-7 cells were seeded in 24-well flat-bottomed microculture plates at a density of 200,000 cells per well, supplied with 1 ml of MEM, and cultured for 72 hr at 37°C. The cells were then washed twice with PBS, 500 µl of reaction buffer (0.1% bovine serum albumin, 0.3 nM [³H]-estradiol, and 1% test compound in MEM containing 0.5% DCC-FBS) was added, and the cells were incubated for 1 hr at 37°C, washed twice with 5 mM PO₄³⁻ containing 0.25 M sucrose and 10% glycerol, and finally treated with 100 µl/well absolute ethanol to extract the hormone. Extracts were counted using a Packard Tri-Carb 4640 spectrometer.

Induction of AlkP Activity

Estrogen-responsive Ishikawa cells (ECACC) were cultured/subcultured as described by the supplier. Cells were plated in 96-well flat-bottomed microculture plates at a density of 12,000 cells per well in phenol-red-free medium supplemented with 5% DCC-FBS, and 24 hr later fresh medium was added followed by test compounds

(initial dilution in DMSO, further dilutions in culture medium). Cells were cultured for 72 hr. To assay AlkP activity [30, 31], the cells were washed with PBS, the plates were inverted, blotted gently on a paper towel, placed at -80°C for at least 15 min, thawed at room temperature for 5–10 min, and then transferred on ice. Next, 50 µl ice-cold solution containing 5 mM p-nitrophenyl phosphate, 0.24 mM MgCl₂, and 1 M diethanolamine (pH 9.8) was added, the cells were warmed to room temperature (time zero), and yellow-colored p-nitrophenol was allowed to accumulate. Cells that received only medium served as negative controls, while cultures treated with 0.1 nM estradiol served as positive controls. The color was monitored every 30 min at 405 nm using the Galaxy 1258 plate reader until positive controls showed an absorbance (A₄₀₅) of about 1.2.

Molecular Modeling

The calculations were performed using the crystal structure of ER β -LBD with genistein, taking into account all of the amino acids within 12 Å from the ligand and the crystallographic water molecule among genistein, GLU305, and ARG346. The structure includes residues from the adjacent subunit, some of which are known to affect binding [8]. His475 was protonated in nitrogen N₂.

The 14 ligand molecules (training and test set) were designed and energy minimized (MM2) using MACROMODEL 6.5 software [32]. The full search in the conformational space was achieved using MM2 force field-Monte Carlo multiple minimum method (MCM) as implemented in MACROMODEL 6.5. One thousand starting conformers were produced and minimized using the TNCG algorithm (rmsG < 0.01 kJ/mol-Å). For the preliminary minimizations of all ligands inside the binding pocket, the AMBER* united atom force field was used with a distance-dependent dielectric "constant" of 4r as implemented in MACROMODEL 6.5. All calculations with MACROMODEL 6.5 software were run on a Silicon Graphics O2 R5000.

For *InRBA* prediction, the PrGen 2.0 program was used. At the beginning, the crystal structure of ER β -LBD with genistein was minimized over 100 steps using PrGen. All molecules were then superimposed over the crystallographic position of genistein as described. Partial atomic charges were attributed using MOPAC 7 (MNDO hamiltonian with NOMM correction) [42]. Solvation energies, entropy corrections, and ligand reference energies were calculated for all ligands after individual Monte Carlo minimization using specific built-in PrGen 2.0 modules. To determine ligand-receptor interaction energy, $E_{\text{ligand-receptor}}$, the program uses the force field Yeti [43]. Binding affinities are obtained by linear regression between *InRBA* and E_{binding} . All calculations with PrGen 2.0 were run on a Silicon Graphics INDY R4500.

Statistics

Data were analyzed using the SPSS 10.0 statistical package for Windows and compared using one-way ANOVA with a Tukey Post Hoc test for multiple comparisons. Differences were considered statistically significant for values of $p < 0.05$. The Pearson's correlation coefficient (r) was used to analyze statistical associations.

Acknowledgments

We thank Drs. P. Chambon and M.G. Parker for plasmids. The technical assistance of Aggela Nastou and Ida Florentin is greatly acknowledged. This work was supported in part by EKBAN66 grant from G.S.R.T-Greece.

Received: July 18, 2003
Revised: December 22, 2003
Accepted: January 5, 2004
Published: March 19, 2004

References

- Adlercreutz, H. (2002). Phyto-oestrogens and cancer. *Lancet Oncol.* 3, 364–373.
- Setchell, K.D., and Cassidy, A. (1999). Dietary isoflavones: biological effects and relevance to human health. *J. Nutr.* 129, 758S–767S.
- Moggs, J.G., and Orphanides, G. (2001). Estrogen receptors: orchestrators of pleiotropic cellular responses. *EMBO Rep.* 2, 775–781.

4. Kuiper, G.G.J.M., Carlsson, B., Gradien, K., Enmark, E., Haggblad, J., Nilsson, S., and Gustafsson, J.A. (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* 138, 863–870.
5. An, J., Tzagarakis-Foster, C., Scharschmidt, T.C., Lomri, N., and Leitman, D.C. (2001). Estrogen receptor beta-selective transcriptional activity and recruitment of coregulators by phytoestrogens. *J. Biol. Chem.* 276, 17808–17814.
6. Brzozowski, A.M., Pike, A.C., Dauter, Z., Hubbard, R.E., Bonn, T., Engstrom, O., Ohman, L., Greene, G.L., Gustafsson, J.A., and Carlquist, M. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* 389, 753–758.
7. Shiau, A.K., Barstad, D., Loria, P.M., Cheng, L., Kushner, P.J., Agard, D.A., and Greene, G.L. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* 95, 927–937.
8. Pike, A.C.W., Brzozowski, A.M., Hubbard, R.E., Bonn, T., Thorsell, A.G., Engstrom, O., Ljunggren, J., Gustafsson, J.E., and Carlquist, M. (1999). Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. *EMBO J.* 18, 4608–4618.
9. Hermanson, O., Glass, C.K., and Rosenfeld, M.G. (2002). Nuclear receptor coregulators: multiple modes of modification. *Trends Endocrinol. Metab.* 13, 55–60.
10. Sanduja, R., Weinheimer, A.J., and Alam M. (1985). Albizoin: isolation and structure of a Deoxybenzoin from the marine mollusc *Merita albicilla*. *J. Chem. Res. Synop.*, 56–57.
11. Wahala, K., and Hase, T.A. (1991). Expedient synthesis of polyhydroxyisoflavonoids. *J. Chem. Soc. Perkin Trans.1* 1, 3005–3008.
12. Kiuchi, F., Chen, X., and Tsuda, Y. (1990). Four new phenolic constituents from Licorise (root of *Glycyrrhiza sp.*). *Heterocycles* 31, 629–636.
13. Beck, A.B., Kowala, C., and Lamberton, J.A. (1966). 2,4-Dihydroxy-4'-methoxybenzil, a probable artefact from formononetin in subterranean clover extracts. *Aust. J. Chem.* 19, 1755–1757.
14. Niwa, K., Hashimoto, M., Morishita, S., Yokoyama, Y., Mori, H., and Tamaya, T. (1999). Preventive effects of *Glycyrrhiza radix* extract on estrogen-related endometrial carcinogenesis in mice. *Jpn. J. Cancer Res.* 90, 726–732.
15. Stevens, M.F.G., McCall, C.J., Lelieveld, P., Alexander, P., Richter, A., and Davies, D.E. (1994). Structural studies on bioactive compounds. 23. Synthesis of polyhydroxylated 2-phenylbenzothiazoles and a comparison of their cytotoxicities and pharmacological properties with genistein and quercetin. *J. Med. Chem.* 37, 1689–1695.
16. Nakanishi, S., Myers, T.C., and Jensen, E.V. (1955). Acid-catalyzed decomposition of chloro- and fluoroformates. *J. Am. Chem. Soc.* 77, 5033–5034.
17. Bolger, R., Wiese, T.E., Ervin, K., Nestich, S., and Checovich, W. (1998). Rapid screening of environmental chemicals for estrogen receptor binding capacity. *Environ. Health Perspect.* 106, 551–557.
18. Maggiolini, M., Bonfiglio, D., Marsico, S., Panno, M.L., Cenni, B., Picard, D., and Ando, S. (2001). Estrogen receptor alpha mediates the proliferative but not the cytotoxic dose-dependent effects of two major phytoestrogens on human breast cancer cells. *Mol. Pharmacol.* 60, 595–602.
19. Vladusic, E.A., Hornby, A.E., Guerra-Vladusic, F.K., Lakins, J., and Lupu, R. (2000). Expression and regulation of estrogen receptor beta in human breast tumors and cell lines. *Oncol. Rep.* 7, 157–167.
20. Monje, P., and Boland, R. (2002). Expression and cellular localization of naturally occurring beta estrogen receptors in uterine and mammary cell lines. *J. Cell. Biochem.* 86, 136–144.
21. Lascombe, I., Beffa, D., Ruegg, U., Tarradellas, J., and Wahli, W. (2000). Estrogenic activity assessment of environmental chemicals using in vitro assays: identification of two new estrogenic compounds. *Environ. Health Perspect.* 108, 621–629.
22. Peterson, G., and Barnes, S. (1996). Genistein inhibits both estrogen and growth factor-stimulated proliferation of human breast cancer cells. *Cell Growth Differ.* 7, 1345–1351.
23. Razandi, M., Pedram, A., Park, S.T., and Levin, E.R. (2003). Proximal events in signaling by plasma membrane estrogen receptors. *J. Biol. Chem.* 278, 2701–2712.
24. Razandi, M., Pedram, A., Greene, G.L., and Levin, E.R. (1999). Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. *Mol. Endocrinol.* 13, 307–319.
25. Migliaccio, A., Castoria, G., Di Domenico, M., de Falco, A., Bilancio, A., Lombardi, M., Bottero, D., Varricchio, L., Nanayakkara, M., Rotondi, A., et al. (2003). Sex steroid hormones act as growth factors. *J. Steroid Biochem. Mol. Biol.* 83, 31–35.
26. Cowley, S.M., and Parker, M.G. (1999). A comparison of transcriptional activation by ER alpha and ER beta. *J. Steroid Biochem. Mol. Biol.* 69, 165–175.
27. Metzger, D., Berry, M., Ali, S., and Chambon, P. (1995). Effect of antagonists on DNA binding properties of the human estrogen receptor in vitro and in vivo. *Mol. Endocrinol.* 9, 579–591.
28. Pettersson, K., Delaunay, F., and Gustafsson, J.A. (2000). Estrogen receptor beta acts as a dominant regulator of estrogen signaling. *Oncogene* 19, 4970–4978.
29. Matsuda, K., Ochiai, I., Nishi, M., and Kawata, M. (2002). Colocalization and ligand-dependent discrete distribution of the estrogen receptor (ER)alpha and (ER)beta. *Mol. Endocrinol.* 16, 2215–2230.
30. Littlefield, B.A., Gurside, E., Markiewicz, L., McKinley, B., and Hochberg, R.B. (1990). A simple and sensitive microtiter plate estrogen bioassay based on stimulation of alkaline phosphatase in Ishikawa cells: estrogenic action of delta 5 adrenal steroids. *Endocrinology* 127, 2757–2762.
31. Markiewicz, L., Garey, J., Adlercreutz, H., and Gurside, E. (1993). In vitro bioassays of non-steroidal phytoestrogens. *J. Steroid Biochem. Mol. Biol.* 45, 399–405.
32. Mohamadi, F., Richards, N.G.J., Guida, W.C., Liskamp, R., Caufield, C., Chang, G., Hendrickson, T., and Still, W.C. (1990). MacroModel: an integrated software system for modeling organic and bioorganic molecules using molecular mechanics. *J. Comput. Chem.* 11, 440–467.
33. Blaney, J., Weiner, P.K., Dearing, A., Kolman, P.A., Jorgensen, E.C., Oatley, S.J., Burridge, J.M., and Blake, J.F. (1982). Molecular mechanics simulation of protein-ligand interactions: binding of thyroid hormone analogues to prealbumin. *J. Am. Chem. Soc.* 104, 6424–6434.
34. Gangloff, M., Ruff, M., Eiler, S., Duclaud, S., Wurtz, J.M., and Moras, D. (2001). Crystal structure of a mutant hERalpha ligand-binding domain reveals key structural features for the mechanism of partial agonism. *J. Biol. Chem.* 276, 15059–15065.
35. Gao, H., Katzenellenbogen, J.A., Garg, R., and Hansch, C. (1999). Comparative QSAR analysis of estrogen receptor ligands. *Chem. Rev.* 99, 723–744.
36. Manolagas, S.C., and Kousteni, S. (2001). Perspective: nonreproductive sites of action of reproductive hormones. *Endocrinology* 142, 2200–2204.
37. Shoker, B.S., Shoker, B.S., Jarvis, C., Clarke, R.B., Anderson, E., Hewlett, J., Davies, M.P., Sibson, D.R., and Sloane, J.P. (1999). Estrogen receptor-positive proliferating cells in the normal and precancerous breast. *Am. J. Pathol.* 155, 1811–1815.
38. Roger, P., Sahla, M.E., Makela, S., Gustafsson, J.A., Baldet, P., and Rochefort, H. (2001). Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumors. *Cancer Res.* 61, 2537–2541.
39. Balasubramanian, S., and Nair, M.G. (2000). An efficient "one pot" synthesis of isoflavones. *Synthetic Communications* 30, 469–484.
40. Denizot, F., and Lang, R. (1986). Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* 89, 271–277.
41. Berthois, Y., Katzenellenbogen, J.A., and Katzenellenbogen, B.S. (1986). Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proc. Natl. Acad. Sci. USA* 83, 2496–2500.
42. Stewart, J.J.P. (1990). A semiempirical molecular orbital program. *J. Comput. Aided Mol. Des.* 4, 1–105.
43. Vedani, A., and Hutha, D.W. (1990). A new force field for modeling metalloproteins. *J. Am. Chem. Soc.* 112, 4759–4767.